Title: **Symplastic communication in organ formation and tissue patterning.**

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Short title: Role of plasmodesmata in organ development

Abstract
Communication between cells is a crucial step to coordinate organ formation and tissue patterning. In plants, the intercellular transport of metabolites and signalling molecules occur symplastically through membranous structures (named plasmodesmata) that traverse the cell wall to connect the cytoplasm and endoplasmic reticulum of neighbouring cells. This review aims to highlight the importance of symplastic communication in plant development. We revisit current literature reporting the effects of changing plasmodesmata in cell morphogenesis, organ initiation and meristem maintenance and comment on recent work involving the identification of novel plasmodesmata regulators and of mobile developmental proteins and RNA molecules. New opportunities for unravelling the dynamic regulation and function of plasmodesmata are also discussed.

Introduction

At the end of the XIX century, plants were thought to be a mere aggregation of isolated cells; however, Eduard Tangl completely shifted the paradigm when he observed cytoplasmic intercellular connections in cotyledons of the tree *Strychnos nux-vomica* [1]. His discovery demonstrated that, despite the cell wall, plant cells communicate to each other and form higher order structures that characterize organisms. Some years later, in 1901, Strasburger named these connections plasmodesmata (PD), etymologically fluid bonds [1,2].

In simple terms, PD are channels made of plasma membrane (PM) that provide cytoplasmic and membranous continuity between neighbouring cells forming the symplasm (Figure 1A). Microscopically these channels appear as concentric cylinders, due to the presence of the desmotubule (DT), a structure derived from endoplasmic reticulum (ER) that becomes trapped in the middle of the channel during cytokinesis [3,4]. Symplastic molecular transport mainly occurs through the cytoplasmic sleeve: the space left between the PM and the DT. Alternative methods for transport can be proposed including diffusion in the ER/DT lumen and lateral segregation of proteins in the PM and ER membrane but their contribution to symplastic intercellular communication is not well defined [5-7] (Figure 1A).

The capacity of molecules to move through PD cytoplasmic sleeve depends on their size and shape and on the cell type and/or developmental stage where they appear. This is because PD number and size exclusion limit (SEL, the maximum molecular

size allowed through any specific pore) are developmentally (and environmentally) regulated [8]. Symplastic molecular transport is extremely important in the phloem, where PD connect companion cells (CC), sieve elements (SE) and the surrounding tissues to regulate communication of metabolites and signals between distant organs [9]. It is also essential in the meristems where transcription factors (and other signalling molecules) move to determine cell fate and tissue development [10,11]. Here we summarize information from recent papers supporting the role of PD in organ formation, meristem development and tissue patterning. Three current topics will be discussed: the regulation of PD during organ development, the role of PD-located receptor proteins in this process and the identification of novel mobile developmental regulators (non-cell autonomous proteins and RNA molecules).

Plasmodesmata regulation during organ formation and vascular patterning

As a key factor in cell-to-cell communication, PD-cytoplasmic aperture is tightly regulated (Figure 1). This is, at least partly, achieved by modifications of the surrounding cell walls, which have different composition (and properties) in the microdomains that are in contact with PD, such as enrichment in certain pectic polysaccharides and callose [4]. Callose (a β-1,3-glucan polymer) is found delimiting PD sites and its accumulation greatly influences transport through the channel by imposing physical constrictions on PD-cytoplasmic aperture (Figure 1B). Callose synthases (CALS) and β-1,3 glucanases (PdBG) that localize at PD sites were identified, providing the machinery for dynamic regulation of callose turnover *in situ*. Altering the expression of these enzymes affects PD transport capacity, thus cell-to-cell symplastic connectivity (Figure 1B). Indeed, gain-of-function mutations in *CALS3* trigger an excessive accumulation of callose at PD impairing root organ development by blocking the transport of transcription factors and miRNAs (such as SHORT-ROOT and microRNA165) that determine the correct formation of the vascular tissue [12]. Further studies using transgenic lines expressing a strongly activated version of CALS3 in specific tissue types and under inducible promoters reveal the importance of callose regulation in the formation of lateral roots and in the transport of hormones involved in vascular patterning and meristem maintenance [12-15]. Characterization of loss-of-function mutations and RNAi lines in CALS10 and CALS7 support the role of callose, and PD, in organ formation and patterning. Mutants in CALS10 (also known as Glucan-Synthase-Like 8 or GSL8) display stomata clustering, presumably

due to the unrestricted mobility of the bHLH transcription factor SPEECHLESS (SPCH) which controls asymmetric cell division in leaves to establish stomata cell fate [16,17]. Separate research, using an inducible RNAi line, identified the role of GSL8 in hypocotyl bending in response to phototropism, a phenotype associated with auxin gradient formation [18]. On the other hand, decreasing CALS7 expression affects the formation of sieve pores: a special type of enlarged PD found at the cell plate of adjacent SE, leading to defective phloem transport, reduced seedling height and aborted embryos among other defects [19,20]. Similarly, a CALS mutant in maize, named *tie-dyed2* (*tdy-2*), is affected in vascular development, specifically in the connections between CC and SE [21]. Correspondingly, phloem export is blocked leading to an increase in the accumulation of starch and sucrose in leaves.

The importance of PdBG in callose regulation during organ formation and vascular patterning was also revealed through the analysis of mutant phenotypes [14,22,23]. Mutants in PdBG1 and PdBG2 (*pdbg1,2*) are affected in root development showing abnormal clustering of lateral root primordia in Arabidopsis [14]. Orthologous genes in *Populus* are regulated by photoperiod, chilling and gibberellins and are involved in the opening of PD for the transport of the FLOWERING LOCUS T (FT), a protein that regulates flowering but also dormancy release at the shoot apex in poplar [24]. PdBG are attached to the PD- PM subdomains by a glycosylphosphatidylinositol (GPI) anchor. These PD- PM subdomains are enriched in sterol and highly glycosylated sphingolipids [25]. Altering this composition, using inhibitors of sterol biosynthesis, was shown to affect PdBG localization, increase callose, decrease symplastic transport and to impair root meristem development.

In addition to callose, other factors/activities can strongly influence PD transport during organ development. Some of these factors emerged from the analysis of the PD proteome [26] while others were identified in independent studies. For example, the GERMIN-LIKE PROTEIN 1 (GLP1), identified by immunoprecipitation with the non-cell autonomous Phloem Protein 16 from *Cucurbita maxima* (CmPP16), was found to regulate PD permeability in Arabidopsis [27]. Expressing tagged versions of these proteins in Arabidopsis (named PDGLP1 and PDGLP2) results in short meristems, reduced primary root growth and increased lateral root length, suggesting defects in the relative distribution of photosynthates between primary and lateral roots [27].

In a separate approach, Vilaine and collaborators reported the identification of NHL26, a phloem-specific protein that localizes to PD [28]. Overexpression (OE) of NHL26 reduces root and seed biomass, increases fresh weight of rosette leaves and delays growth, senescence and flowering [28]. These plants also accumulate sugars in mature source leaves which correlate with a reduction of these metabolites in phloem sap. The evidences suggest a function for NHL26 in controlling sugar export between CC and the SE through regulating PD permeability [27].

Also influencing phloem export, overexpression of the PD-located rice gene Grain Setting Defect1 (GSD1) causes sugar accumulation in leaves and reduces panicle size and grain setting [29]. GSD1 encodes a putative remorin, which are plant-specific proteins of unknown function that appear attached to PM lipid-raft domains and enriched around PD in Solanaceae [30]. Remorins encode conserved coiled-coil domains presumably involved in protein-protein interactions. The research indicates that GSD1 interacts with other PD proteins, including Actin 1 (ACT1), to regulate PD permeability and phloem transport.

Another protein influencing phloem transport is the choline transporter-like protein, CHER1 [31]. CHER1 localizes to the trans-Golgi network, the cell plate and polarly at the incipient sieve plates during early SE differentiation. The mutant *cher1* displays a short root phenotype characterized by a short meristem size. This is accompanied by blocked connectivity between the protophloem and the root meristem, discontinuous phloem differentiation, longer retention of the desmotubule in the pores, reduced sieve plate area and decreased pore density [31]. The mechanism underlying these effects is unknown but the results point to CHER1 as one of the major regulators in sieve pore formation, a process that involves PD modifications.

Taken together the research demonstrates the importance of PD regulation in organ formation, vascular patterning and in the phloem transport of resources that determine the development of sink (developing) and source (photosynthetic) tissues. The mechanisms controlling PD aperture during organ formation and vascular patterning are mostly unknown but require the participation of multiple proteins, that either alone or assembled, modify PD structure, transport capacity and/or their differentiation into sieve pores.

Receptor proteins act at plasmodesmata to regulate organ development

Research on receptor proteins that target and/or interact at PD to function in developmental signalling is gaining momentum [32]. Analysis of the PD proteome in Arabidopsis identified three receptor-like kinases (RLKs) and a number of receptor-like proteins including the PD-Located Proteins (PDLPs) and the chitin receptor-like protein LYM2 [26 ,32]. In rice, a candidate gene approach was undertaken to investigate the cell wall proteome leading to the identification of 15 putative RLKs, six of which were confirmed to target PD using fluorescent tagging assays [33].

Although most of the research in PD-located receptor proteins focuses on their function in plant-pathogen interactions, new cumulative data support their role in development. For example, PDLP overexpression impairs plant growth and recent work links this receptor to the regulation of callose at PD [34-36].

The Arabidopsis RLK protein STRUBBELIG (SUB), involved in organ formation and tissue morphogenesis, also localizes at PD, where it physically interacts with the protein QUIRKY (QKY) to act non-cell autonomously in the regulation of flower shape, leaf symmetry and integument development [37]. QKY homolog FT-INTERACTING PROTEIN 1 (FTIP1) also accumulates at PD in phloem cells and regulates the transport of the flowering signal FT [38].

Interaction of receptor proteins is also proposed as a mechanism to regulate the PD transport of factors maintaining stem cell fate in the apical meristems (Figure 2). In the shoot apical meristem (SAM), the receptor protein CLAVATA 1 (CLV1) is activated by the small peptide CLV3 which act as a signalling ligand to regulate the expression domain of the mobile stem cell transcription factor WUSCHEL (WUS) [39,40]. In the root meristem, the PD-located receptor kinase CRINKLY4 (ACR4 in Arabidopsis) interacts with CLV1 upon perception of the CLV3-like peptide CLE40p to trigger differentiation of the columella [41]. Although the mechanism is unknown, the results suggest that the formation of ACR4/CLV1 complexes restrict the movement of developmental regulators (WUS-like factors) necessary to maintain stem cell fate [41]. This elegant research highlights the similarities in the mechanisms that regulate intercellular signalling, stem cell maintenance and differentiation in roots and shoots and the importance of PD-located receptor proteins in this process. The model proposed in Figure 2 aims to illustrate this idea: signals that move in the apoplast bind specific receptors that interact at PD to modulate the symplastic transport of proteins that determine cell fate during organ development. Work undertaken by different research groups aims to identify the

signalling ligands, the receptors proteins and the mobile factors involved in this mechanism.

Identification and developmental function of novel mobile proteins and RNAs

Defects in PD structure and connectivity restrict the cell-to-cell transport of transcription factors and a range of RNA molecules (messenger RNAs (mRNAs), short interference RNAs (siRNA), microRNAs (miRNAs) and trans-acting siRNAs (TasiRNAs)) that coordinate development (for a recent review consult [42-44]). The list of transcriptional and signalling factors likely transported through PD is continuously growing (Table 1). Reports demonstrating the requirement of proper PD regulation for the transport of well characterized transcription factors, such as SHORTROOT (SHR) [12,45-47], are added to new studies suggesting the intercellular mobility of other important developmental regulators, such as ANGUSTIFOLIA3 (AN3) which moves from the mesophyll to control epidermal cell proliferation in leaves [48]. A genomic screen of transcription factors in Arabidopsis identified 22 mobile factors distributed within the homeobox, GRAS, and MYB families [49]. Further characterization of the Dof transcription factor AtDof4.1, isolated in this screen, supports intercellular transport through PD and identified a small motif sufficient to confer mobility to otherwise cell-autonomous proteins [50]. New evidence also suggests the intercellular mobility of PLETHORA2 (PLT2), an auxin-induced transcription factor necessary to establish the different developmental domains in Arabidopsis root [51]. Using a combination of modelling and experimental approaches, the authors propose that intercellular movement of PLT2, and dilution of its concentration due to root growth, are both necessary to generate a longitudinal gradient that defines root zonation [51]. Related to vascular development, Zhou et al. (2013) identified two new transcription factors (AT-HOOK MOTIF NUCLEAR LOCALIZED PROTEIN 3 and 4, AHL3/AHL4) that move and interact in the stele to regulate non-cell autonomously the development of the xylem [52].

More knowledge has been gathered on the structural requirements for protein trafficking through PD. In the SAM, the transport of WUS was found to be an intrinsic property (independent of the cellular context) that is coded in multiple domains of the protein [40]. Conversely, the intercellular transport of the maize protein KNOTTED1 (KN1), and its ortholog in Arabidopsis STM, involved in SAM maintenance, is determined by specific signatures present in the homeodomain sequence [53,54].

Concretely, two evolutionary conserved surface residues, an arginine and a leucine, were found essential for intercellular transport [54]. In addition, a chaperonin complex was identified as crucial to refold homeodomain proteins after translocation and this mechanism also seems involved in regulating the transport of viral movement proteins [55,56].

The importance of mobile RNA molecules in tissue patterning and organ development emerged from recent publications. Thousands of transcripts moving in the phloem of Arabidopsis and other plant species were identified using different strategies (such as grafting, translocation of RNA between host and parasitic plants and phloem sap analysis) [57-61]. These phloem RNA molecules move long-distances and between CC and SE, by diffusion or in complex with RNA-binding proteins, to regulate development in target tissues. However questions remain regarding the selectivity for phloem translocation or the final destination of these transcripts [57]. The transport of miRNA and siRNA molecules can also be phloem independent [62]. Research on miR394 suggests that it moves from the L1 layer of the SAM to the inner stem cell layers to repress *LEAF CURLING RESPONSIVENESS* (*LCR*, a gene involved in leaf and shoot meristem development) acting as a positional cue to maintain shoot stem cell activity [63,64]. Also important for patterning, miR165/166 move between cell layers in embryos and root meristem to regulate *CLASS III HOMEODOMAIN LEUCINE ZIPPER* (*HD-ZIP III*) proteins [47,65]. In turn, tasiR-ARF, a trans-acting siRNA that targets *AUXIN RESPONSE FACTOR 3* (*ARF3*) and *ARF4*, diffuses from the adaxial to the abaxial side to establish leaf polarity [62,66].

siRNA can also move long-distances in a phloem-independent pathway from root to shoot [67]. When produced in roots, siRNA moves to the shoot by a combination of short-range cell-to-cell communication events and amplification of the signal in all cells *en route*. Interestingly, the spreading of the silencing signal is affected in mutants in a hydrogen peroxide (H_2O_2)-producing type III peroxidase (named RCI3) concordant with previous research indicating the role of H_2O_2 on regulating PD permeability [68]. Together, the findings support the involvement of PD in the transport of siRNA and their role in developmental signalling [67].

Conclusions and future perspectives

The role of PD in organ development and tissue patterning is now well-established. However, knowledge on the mechanisms regulating PD structure and transport capacity and on the specific signatures/modifications that determine the mobility of developmental factors is still sparse. Protein-protein interactions at PD sites and crosstalk between the symplastic and the apoplastic pathway for molecular transport are proposed to occur in the apical meristems to regulate stem cell fate and organogenesis. Similar mechanisms might play a role in other developmental and morphogenetic processes such as the post-embryonic initiation of secondary meristems and meristemoids.

An effective pathway to regulate development is through modifications in hormonal transport. PD mediates the phloem- transport of cytokinins [14] but their contribution to the establishment of auxin gradients is still under discussion. Research using moss and a combination of computational and experimental approaches, has shed some light on this conundrum [69]. In *Physcomitrella,* bi-directional diffusion of auxin through PD is required to generate realistic branching patterns *in silico* and altering PD connectivity (by inhibition of callose) is sufficient to inhibit branching *in vivo*. How conserved is this mechanism in higher plants is still unknown but mosses emerged from these studies as a suitable system to understand PD roles in development [70]. Independent lines of research demonstrate the importance of PD in regulating auxin response *in planta*. Grafting experiments between Arabidopsis and *Nicotiana benthamiana*, showed that Aux/IAA transcripts (key regulators of auxin-responsive genes) move from mature leaves to roots, to regulate the initiation of lateral roots [71]. In addition, the phloem transport of Cyclophilin 1 (Cyp1) from a wildtype scion to a mutant rootstock restores auxin signalling and lateral root development in the tomato *diageotropica* (*dgt*) mutant which is normally auxin insensitive. This effect is dependent on light intensity suggesting that movement of Cyp1 can be involved in coordinating shoot-root relations in response to the plant photosynthetic status [72].

Experimental approaches combined with predictions from mathematical simulations are useful for determining the significance of sRNA and protein movement in developmental signalling. Using these approaches, it was shown that a gradient in the distribution of miR165/166 can be translated into sharp boundaries in the expression of its target PHABULOSA (PHB) to determine vascular pattern [73]. Computational models to predict the importance of PD in the establishment of small molecular gradients have also been formulated but quantitative data to test these

models are still missing [44,68]. Advances in microscopy techniques to determine PD transport parameters, the phenotypic analysis of mutants in PD form and function and the discovery of tools to modify PD permeability will be essential to make progress in this field.

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Table 1. List of mobile proteins (blue shaded cells) and sRNAs (in green) studied in the last five years with a function in organ development and patterning.

Mobile factor[1]	Direction of movement	Function	References[2]
AHL3/AHL4	From procambium cells to the xylem	Xylem specification	[52]
PLT2	Longitudinally from the root meristem forming a gradient	Longitudinal root zonation	[51],[74]
SHR	From the stele into the endodermis	Ground tissue formation	[12],[45],[46]
WUS	From the organizing centre to L1, L2 layers in the shoot	Meristem maintenance	[40]
FT	From leaf and cotyledons to the SAM	Transition to flowering	[38]
SPCH	Cell-to-cell diffusion in the leaf epidermis of *chorus*	Stomata cell fate	[16]
AtDof4.1	From pericycle to endodermis/cortex in roots	Unknown	[50]
KN1/STM	Broadly in the SAM	Meristem maintenance	[54]
AN3	From the mesophyll to the epidermis in leaves	Leaf development	[48]
Cyp1	From leaves to root in tomato	Regulation of root growth	[70]
miR165/6	From endodermis into the stele	Xylem specification	[47],[65]
miR394	From L1 into inner layers in the shoot meristem	Meristem maintenance	[63,64]
tasiR-ARF	From the adaxial to the abaxial side of the leaf	Establishment of leaf polarity	[66]
IAA18 and IAA28 mRNA	From mature leaves to roots	Lateral root formation	[69]
Artificial siRNA	From root to shoot	Gene silencing	[75]

[1]Full name of mobile factors is provided in the text.
[2]Citations are included in the reference list.

Figure Caption

Figure 1. Transport pathways and PD regulation by callose. (A) Intercellular transport occur through PD cytoplasmic sleeve (black arrows), by diffusion in the lumen of the endoplasmic reticulum (ER) and the desmotubule (DT) (orange arrows) and, potentially, by lateral segregation in the membranes (green discontinuous arrows). Plasma membrane (PM), cell wall (CW) and PD-cytoplasmic aperture (in discontinuous blue) are indicated. (B) PD transport is regulated by the deposition of callose in the surrounding cell wall. Callose is produced at PD sites from UDP-glucose by callose synthases (CALS) and degraded to glucose subunits by PD-located beta-1,3 glucanases (PdBG). Callose turnover depends on the activity of these enzymes. High levels of callose restricts PD-cytoplasmic aperture blocking molecular transport thus cell-to-cell symplastic connectivity.

Figure 2. Hypothetical model that illustrates the role of receptor proteins in regulating PD connectivity and stem cell differentiation. Open PD allows the transport of developmental proteins such as transcription factors involved in stem cell fate specification. Upon perception of signalling molecules specific PD-located receptors (such as ACR4) and membrane RLKs (such as CLV1) relocate at PD proximity and form complexes that trigger a cascade of unknown events (discontinuous arrows) that either modify protein movement capacity or PD aperture (potentially through changing callose as in Figure 1B). Restricted intercellular communication (in red) of stem cell factors leads to the activation of the stem cell differentiation program.

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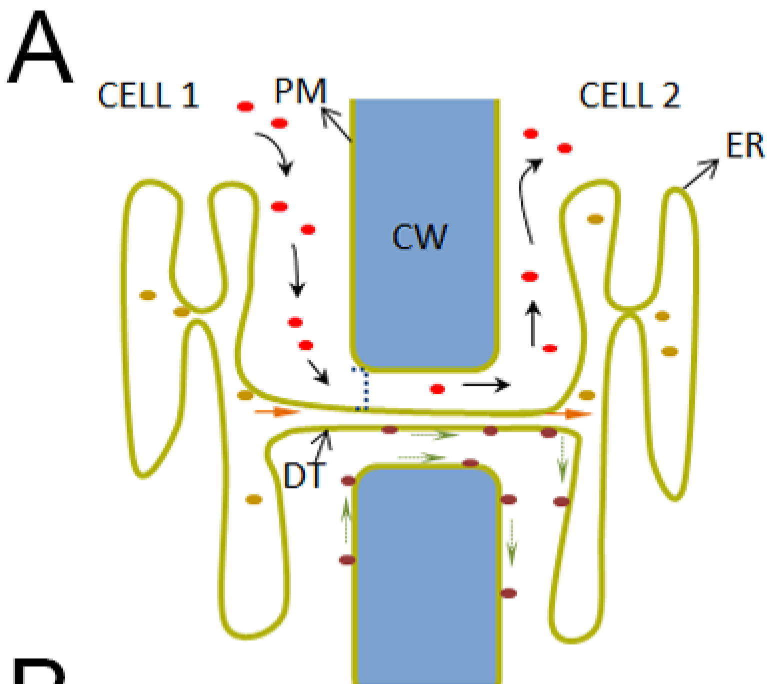
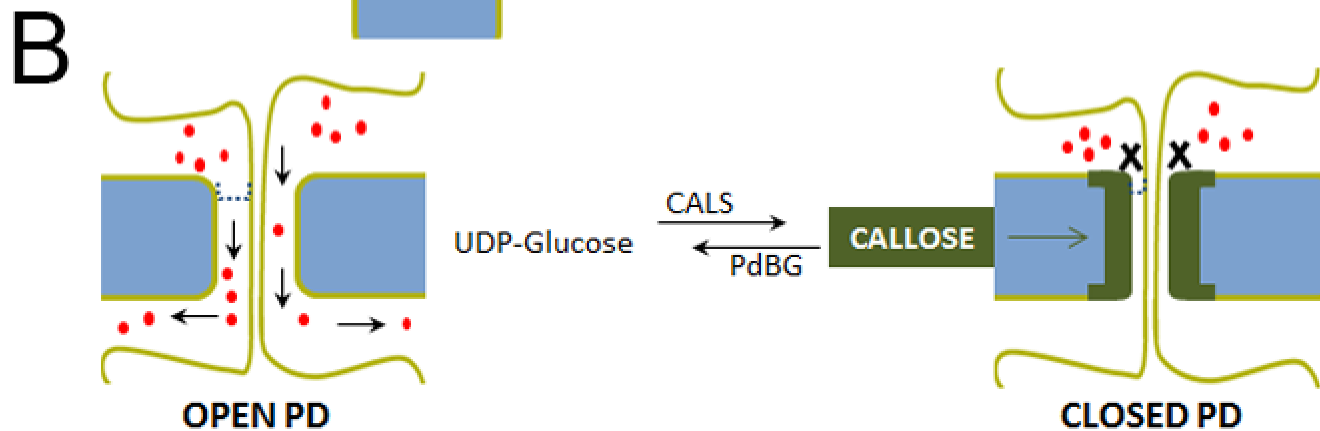

A
CELL 1
PM
CELL 2
ER
CW
DT
Molecular transport through cytoplasmic sleeve
Diffusion of small molecules in the ER/DT lumen
Diffusion of membrane proteins
PD-cytoplasmic-aperture


B
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CALLOSE
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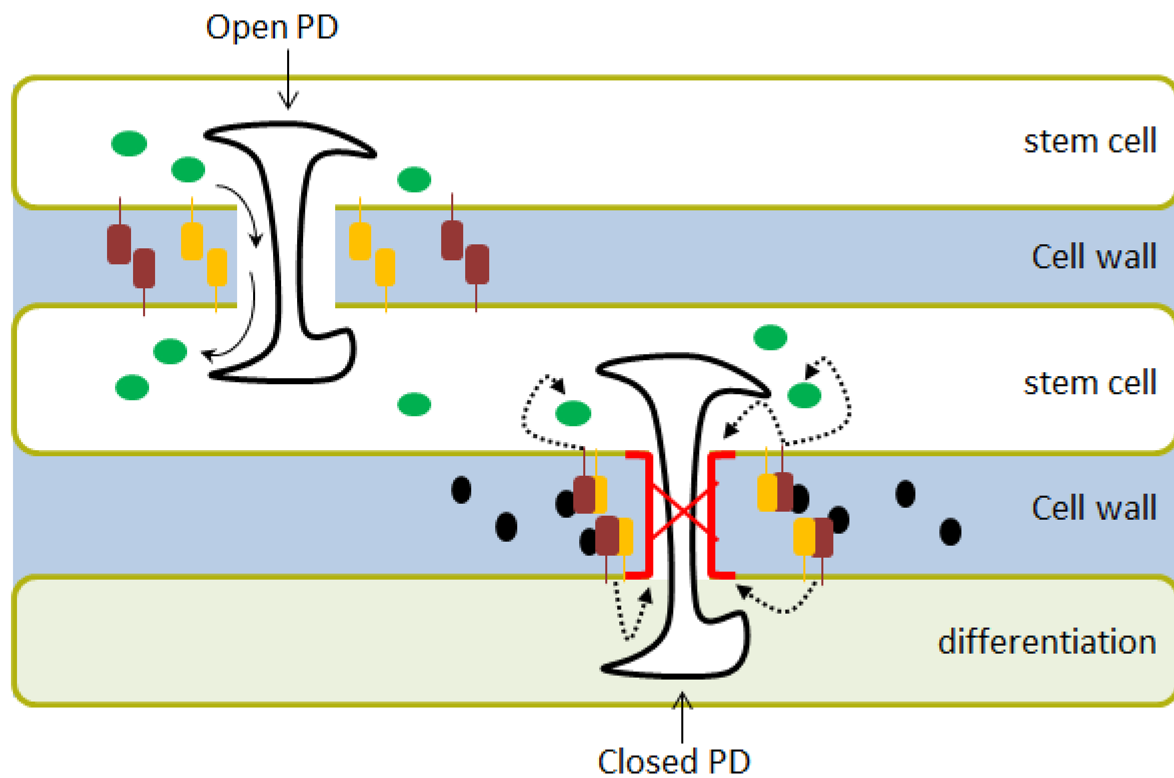
CLOSED PD


Open PD
stem cell
Cell wall
stem cell
Cell wall
differentiation
Closed PD
Developmental proteins
PD-located receptor
Membrane RLK
Signalling molecules